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## INTRODUCTION:

In order to enhance the immune response against breast cancer, we proposed to introduce the HLA-A2 binding peptide, KIFGSLAFL, derived from breast cancer associated antigen HER-2/neu, to the dermal Langerhans cells (LC), to stimulate the cytotoxic T-lymphocyte (CTL) response to breast cancer cells. The peptide or the minigen encoding it are to be transported across the skin by transdermal electroporation, which can cover a large skin area. We proposed to optimize the electric parameters for maximum delivery, to monitor both the migration of LC after exposure, and the resulting CTL response to this peptide antigen.

## BODY:

### 1. Measurement of the molecular weight limit of transdermal delivery technology:

The efficiency of transdermal delivery by electroporation was tested using FITC-labeled Dextrans of different molecular weights. Excised porcine epidermis or murine skin was placed in between two chambers of a Vertical Diffusion Holder (figure. 1). We found that those charged molecules with molecular weight less than 1,000 could be transported through the skin efficiently. The flux was in the order of  $1\text{--}10\text{ }\mu\text{g}/\text{cm}^2/\text{min}$  when 1 msec pulses of 100V were applied to the skin at 1 Hz. This is similar to the flux we measured in live mice (Johnson et al., 1998, 2002). Molecules at a higher molecular weight were transported at a lower flux, which dropped off significantly when molecular weight exceeded 10,000 (Sen et al., 2002). Applying anionic lipids to the skin during pulse application enhanced the transport flux and extended the molecular weight limit for efficient transport to beyond 4,000 (figure 2). However, transport of molecules with molecular weights at and beyond 10,000 still posted difficulty. This finding agreed with those reported recently by Lombry et al., (2000). With this finding, transdermal transport of minigenes would be a problem. We had to restrict the delivery to that of the antigenic peptide alone (MW~9,000).

### 2. Synthesis and optimization of delivery of antigenic peptides:

The HLA-A2 binding HER-2/neu peptide, KIFGSLAFL, and as a control, the K<sup>b</sup>-binding OVA peptide, SIINFEKL, were

synthesized and purified by the Biopolymer Facility of this Institute. In order to measure the quantity of peptides that can be delivered transdermally by electroporation, some of the OVA peptide was labeled with rhodamine. This peptide carries 1 net negative charge when the lysine is linked to a rhodamine. The transport across excised murine skin was measured using a Vertical

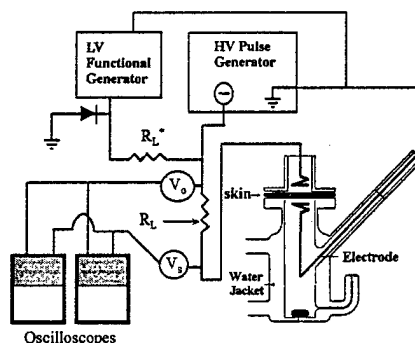


Figure 1. Schematic drawing of the Vertical Diffusion Holder and electrode arrangement.

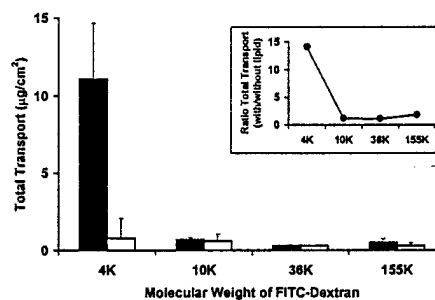


Figure 2. Transport of FITC-Dextrans of different molecular weights after 1 min of electroporation (100V, 1ms pulse width at 1Hz) with (■) and without (□) anionic lipids. Inset: Plot of the enhancement ratio of the total transport with and without added lipid.

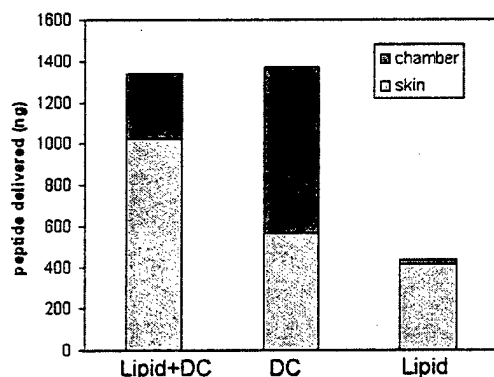


Figure 3. Transport of the SIINFEKL-rhodamine peptide to murine skin, in the presence or absence of anionic lipids or a 9VDC potential after fifteen 1 msec pulses of 100V were applied at 1 Hz.

Diffusion Holder as described above. The donor chamber contains the cathode while the receiver chamber contains the anode. The amount of peptide that could be delivered to and through the skin was about  $1 \mu\text{g}/\text{cm}^2$  after fifteen 1 msec pulses of 100V were applied at 1 Hz. An undetermined portion of the labeled peptide was visibly retained by the remaining hair even after clean shaving. Application of anionic lipids tended to encourage the peptide to remain in the skin rather than passing through to the receiving chamber (figure 3). Application of a 9VDC potential had the opposite effect. Labeling the HER-2/neu peptide using the lysine residue would result in neutralizing the peptide, thereby losing the electrophoretic transport advantage of both the pulse and DC potential. Without labeling, the transport of this peptide was difficult to measure.

3. Design of transdermal delivery electrodes: In order to maximize the transdermal delivery of antigenic peptides to murine skin *in vivo*, a special electrode was designed and used. The electrode was modified from a commercial Biopotential Skin Electrode E256A (In Vivo Metric, Ukiah, CA). The cavity in front of the AgCl electrode was filled with highly conductive Sigma Gel (Parker Laboratory, Orange, NJ). 20  $\mu\text{l}$  of the peptide to be delivered was soaked onto a filter disc and placed in between the skin surface and the gel-filled electrode. The skin surface was cleanly shaved and locally wetted with conducting buffered solution (100 mM NaCl, 10 mM TRIS pH. 7.4) and anionic lipids when needed. Placing concentrated peptides closer to the skin surface increased the delivery by an order of magnitude, as compared to filling the electrode cavity with diluted peptide in buffered solution. This design was first tested *in vitro* with a Vertical Diffusion Holder, then *in vivo* with mice.

4. Breeding of HLA-A2/Kb transgenic mice: The study of the CTL response to the HLA-A2 binding HER-2/neu peptide demands the use of HLA-A2/K<sup>b</sup> transgenic mice. Breeding pair of HLA-A2/Kb transgenic mice were purchased through the Department of Laboratory Animals of this Institute. A successful breeding program has resulted in a constant supply of over 15 mice at any given time. Because of the expenses of transgenic mice, initial quantitative measurement of peptide delivery was done *in vitro* using Balb-c mouse skin in a Vertical Diffusion Holder.

5. Measurement of CTL response after immunization of the antigenic peptide: The HLA-A2 binding peptide, KIFGSLAFL, was delivered to HLA-A2/K<sup>b</sup> transgenic mice as a vaccine, and the CTL response of the immunized mice was monitored. For each experiment, 4 mice received peptide delivery by transdermal electroporation. The delivery and reference electrodes were placed 25 mm apart, on the shaved back skin of the mouse. 100  $\mu\text{g}$  of the peptide in 20  $\mu\text{l}$  was soaked onto a filter disc and placed in between the skin surface and the gel-filled delivery electrode. The reference electrode was of the same construction but was connected to the opposite polarity and with a buffer-filled filter disc instead of a peptide-filled one. The KIFGSLAFL peptide has a net positive charge and was placed under the anode for delivery. The

skin surface was locally wetted with conducting buffered solution (100 mM NaCl, 10 mM TRIS pH. 7.4) to ensure good electric contact. Mice were anesthetized before application of electric pulses. 20 pulses at 250V (125V per skin passage) and 1 msec duration were applied at 1 Hz. A DC potential of 9V was applied concurrently and continued for 10 min after the cessation of pulses. As positive control, 2 mice were injected i.d. with the same amount of peptide together with Floyd complete adjuvant and a 20-mer peptide T-helper promoter. Booster delivery was made after 14 days but with incomplete adjuvant. 2 untreated mice served as negative control. 14 days after booster delivery, mice were sacrificed, and spleenocytes were removed for CTL assay. The more sensitive ELISPOT assay (Manjili et al., 2002) was used instead of the originally proposed chromate release assay for CTL response. In brief, spleenocytes were dissociated and stimulated with 1  $\mu$ g/ml of the peptide (or con-A for positive control) and cultured on 96-well filter plates for 20 hr. After repeated washing, the filter plates were labeled with biotinylated IFN- $\gamma$  antibody. The plates were developed in alkaline phosphatase avidine-D and BCIP/NBT. The spots per well were counted under a microscope. So far, mice immunized by electroporation have shown no significant CTL response. We suspect that either the peptide delivered was insufficient, or the adjuvants in transdermal electroporation were absent because of molecular weight limitation. To address the first concern, we plan to reduce stepwise the amount of i.p. injected peptide to 1  $\mu$ g to determine the threshold for CTL response, and to match that by electroporation. The second concern will be addressed by combined peptide electroporation and i.p. injection of adjuvants. In view of recent report successes of immunization by transdermal electroporation without adjuvants (Misra et al., 2000; Upadhyay, 2001), using diphtheria toxoid and a peptide vaccine against hepatitis B, we are confident that the delivery of the peptide vaccine KIFGSLAFL against breast cancer cells will succeed eventually.

#### KEY RESEARCH ACCOMPLISHMENTS:

- Measurement and extension of the upper molecular weight limit of transdermal delivery by electroporation.
- Optimized conditions for delivering antigenic peptides to the skin by electroporation.
- Improved electrode design for optimal peptide delivery.
- Delivered the HER-2/neu derived peptide, KIFGSLAFL, to HLA-A2/K<sup>b</sup> transgenic mice by transdermal electroporation.

#### REPORTABLE OUTCOMES:

#### CONCLUSIONS:

Successful delivery of the HER-2/neu derived peptide vaccine, KIFGSLAFL, to a large population of skin Langerhans cells (LC) by transdermal electroporation is expected to enhance the CTL response to breast cancer cells. To enable the delivery of large molecules, the upper molecular weight limit of transdermal delivery of macromolecules by electroporation has been extended by using anionic lipids, to <10,000. This enables the delivery of antigenic peptides but not minigenes. Using either a skin chamber or live mice, we measured the transdermal flux of antigenic peptides (M.W.~9,000) to be in the order of 1-10  $\mu$ g/cm<sup>2</sup>/min, when 1 msec pulses of 100V were applied to the skin at 1 Hz. CTL response to delivery of the peptide vaccine KIFGSLAFL has so far been negative. We attribute the lack of response to either that the peptide

delivered was insufficient, or that the adjuvants in transdermal electroporation were absent because of molecular weight limitation. Increase the amount of delivery, with or without co-injection of adjuvant, could overcome the problem.

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